DNA VACCINE AGAINST PROLIFERATING ENDOTHELIAL CELLS AND METHODS OF USE THEREOF

GOVERNMENTAL RIGHTS

This invention was made with government support under Contract No. 5-70373-COLON by the National Institutes of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

This invention relates to deoxyribonucleic acid (DNA) vaccines encoding suitable molecules effective for eliciting an immune response against proliferating endothelial cells. More particularly this invention relates to DNA vaccines encoding for the vascular endothelial growth factor (VEGF) receptor. This invention also relates to methods of using the DNA vaccine to inhibit vascular endothelial cell proliferation, tumor growth, and angiogenesis.

BACKGROUND OF THE INVENTION

Vaccines have been utilized to provide a long term protection against a number of disease conditions by very limited administration of a prophylactic agent that stimulates an organism's immune system to destroy disease pathogens before they can proliferate and cause a pathological effect. Various approaches to vaccines and vaccinations are described in Bernard R. Glick and Jack J. Pasternak, *Molecular Biotechnology, Principles and Applications of Recombinant DNA*, Second Edition, ASM Press pp. 253-276 (1998).

Vaccination is a means of inducing the body's own immune system to seek out and destroy an infecting agent before it causes a pathological response. Typically, vaccines are either live, but attenuated, infectious agents (virus or bacteria) or a killed form of the agent. A vaccine consisting of a live bacteria or virus must be non-pathogenic. Typically, a bacterial or viral culture is attenuated (weakened) by physical or chemical treatment. Although the agent is nonvirulent, it can still elicit an immune response in a subject treated with the vaccine.

An immune response is elicited by antigens, either specific macromolecules, or an infectious agent. These antigens are generally either proteins, polysaccharides, lipids, or glycolipids, which are recognized as "foreign" by lymphocytes known as B cells and T cells. Exposure of both types of lymphocytes to an antigen elicits a rapid cell division and differentiation response, resulting in the formation of clones of the exposed lymphocytes. B cells produce plasma cells, which in turn, produce proteins called antibodies (Ab), which selectively bind to the antigens present on the infectious agent, thus neutralizing or inactivating the pathogen (humoral immunity). In some cases, B cell response requires the assistance of CD4 helper T cells.

The specialized T cell clone that forms in response to the antigen exposure is a cytotoxic T lymphocyte (CTL), which is capable of binding to and eliminating pathogens and tissues that present the antigen (cell-mediated or cellular immunity). In some cases, an antigen presenting cell (APC) such as a dendritic cell, will envelop a pathogen or other foreign cell by endocytosis. The APC then processes the antigens from the cells, and presents these antigens in the form of a histocompatibility molecule:peptide complex to the T cell receptor (TCR) on CTLs, thus stimulating an immune response.

Humoral immunity characterized by the formation of specific antibodies is generally most effective against acute bacterial infections and repeat infections from viruses, whereas cell-mediated immunity is most effective against viral infection, chronic intracellular bacterial infection, and fungal infection. Cellular immunity is also known to protect against cancers and is responsible for rejection of organ transplants.

Antibodies to antigens from prior infections remain detectable in the blood for very long periods of time, thus affording a means of determining prior exposure to a pathogen. Upon re-exposure to the same pathogen, the immune system effectively prevents reinfection by eliminating the pathogenic agent before it can proliferate and produce a pathogenic response.

The same immune response that would be elicited by a pathogen can also sometimes be produced by a non-pathogenic agent that presents the same antigen as the pathogen. In this manner, the subject can be protected against

subsequent exposure to the pathogen without having previously fought off an infection.

Not all infectious agents can be readily cultured and inactivated, as is required for vaccine formation, however. Modern recombinant DNA techniques have allowed the engineering of new vaccines to seek to overcome this limitation. Infectious agents can be created that lack the pathogenic genes, thus allowing a live, nonvirulent form of the organism to be used as a vaccine. It is also possible to engineer a relatively nonpathogenic organism such as *E. coli* to present the cell surface antigens of a pathogenic carrier. The immune system of a subject treated with such a transformed carrier is "tricked" into forming antibodies to the pathogen. The antigenic proteins of a pathogenic agent can be engineered and expressed in a nonpathogenic species and the antigenic proteins can be isolated and purified to produce a "subunit vaccine." Subunit vaccines have the advantage of being stable, safe, and chemically well defined; however, their production can be cost prohibitive.

A new approach to vaccines has emerged in recent years, broadly termed genetic immunization. In this approach, a gene encoding an antigen of a pathogenic agent is operably inserted into cells in the subject to be immunized. The treated cells are transformed and produce the antigenic proteins of the pathogen. These *in vivo*-produced antigens then trigger the desired immune response in the host. The genetic material utilized in such genetic vaccines can be either a DNA or RNA construct. Often the polynucleotide encoding the antigen is introduced in combination with other promoter polynucleotide sequences to enhance insertion, replication, or expression of the gene.

DNA vaccines encoding antigen genes can be introduced into the host cells of the subject by a variety of expression systems. These expression systems include prokaryotic, mammalian, and yeast expression systems. For example, one approach is to utilize a viral vector, such as vaccinia virus incorporating the new genetic material, to innoculate the host cells. Alternatively, the genetic material can be incorporated in a vector or can be delivered directly to the host cells as a "naked" polynucleotide, i.e. simply as purified DNA. In addition, the DNA can be stably transfected into attenuated

bacteria such as *Salmonella typhimurium.* When a patient is orally vaccinated with the transformed *Salmonella*, the bacteria are transported to Peyer's patches in the gut (i.e., secondary lymphoid tissues), which then stimulate an immune response.

DNA vaccines provide an opportunity to immunize against disease states that are not caused by traditional pathogens, such as genetic diseases and cancer. Typically, in a genetic cancer vaccine, antigens to a specific type of tumor cell must be isolated and then introduced into the vaccine. An effective general vaccine against a number of cancers can thus entail development of numerous individual vaccines for each type of cancer cell to be immunized against.

One general approach to treatment of tumors involves administering angiogenesis inhibiting compounds to patients with growing tumors. Angiogenesis is the process by which new capillaries and blood vessels form. Angiogenesis is important in embryonic development, tissue growth, tissue repair, and tissue regeneration. In addition to these normal and essential processes, angiogenesis is also involved in many abnormal pathological processes such as tumor growth, tumor metastasis, and ocular vascular diseases such as diabetic retinopathy.

Angiogenesis involves a number of interdependent processes, including (a) activation of vascular endothelial cells, (b) decomposition of cell matrix proteins by endothelial cells expressing protease activity, (c) migration of endothelial cells to a potential growth sites, (d) proliferation of endothelial cells and (e) tube formation by differentiation of endothelial cells. Each of these processes is affected by a variety of promoter substances such as fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factors (VEGF). The vascular endothelial growth factors (collectively VEGF) play a crucial role in endothelial cell growth and differentiation. VEGF acts by binding to receptor protein-tyrosine kinases present in the endothelial cell membranes, which in turn initiate a cascade of signal transduction reactions that stimulate cell growth.

Inhibition of pathological angiogenesis has been proposed as a treatment for tumors. See, for example, Folkman *et al. Science,* **221**, 719, (1983). The basic concept of such treatment is that, since tumors require vascularization to grow, inhibition of blood vessel formation, through the administration of angiogenesis inhibiting compounds, will prevent tumor growth by starving the tumor of its blood supply. A disadvantage of his approach is that angiogenesis inhibitors must be administered on a relatively continuous basis to prevent tumor growth. A cessation in delivery of the inhibitor can lead to a resumption of tumor growth. A vaccine effective at inhibiting angiogenesis would be an attractive preventative agent against tumor formation.

There is a continuing need for a generally effective vaccine for immunization against angiogenesis, which can also inhibit the growth of a variety of tumors without the need for targeting specific tumor antigens. The present invention satisfies this need.

SUMMARY OF THE INVENTION

A DNA vaccine effective for inhibiting endothelial cell proliferation comprises a DNA construct that operably encodes a VEGF receptor protein. The DNA vaccine comprises a polynucleotide that encodes a receptor protein for VEGF, such as VEGFR-2 (KDR; SEQ ID NO: 2), VEGFR-1 (Flt-1; SEQ ID NO: 4), and Flk-1 (SEQ ID NO: 6; the murine homolog of KDR), e.g., DNA sequences SEQ ID NO: 1, SEQ ID NO: 3, and SEQ ID NO 5, respectively. The vaccine can comprise a linear nucleic acid such as a purified DNA construct, or a DNA construct incorporated in a plasmid vector. The DNA vaccines of the present invention stimulate formation of CTLs active against proliferating endothelial cells that overexpress VEGFR-2.

Endothelial cells form the lining of mammalian vascular tissue. The proliferation of endothelial cells is a key process in angiogenesis. The vaccines of the present invention provide a method for producing long term inhibition of angiogenesis in an organism treated with the vaccine by eliciting an immune response against proliferating endothelial cells. Non-proliferating endothelial cells, such as the linings of established blood vessels, do not present

significant quantities of VEGF receptor antigens and thus remain substantially unaffected by the CTLs that are produced in response to the vaccine.

In a method aspect of the present invention, a DNA vaccine is utilized to provide long term inhibition of endothelial cell proliferation in a vaccinated patient. In one method embodiment, a DNA vaccine comprising a polynucleotide construct operably encoding a VEGF receptor protein is administered orally to a patient in need of inhibition of endothelial cell proliferation in an amount that is sufficient to elicit an immune response against proliferating endothelial cells.

The present invention also provides a method of inhibiting angiogenesis in a patient vaccinated with a DNA vaccine. In such a method embodiment, an immune response eliciting amount of a vaccine that includes a DNA construct operably encoding a VEGF receptor protein is administered to a patient suffering from an angiogenesis-related disease.

In yet another method aspect of the present invention, tumor growth is inhibited by vaccinating a patient with a DNA vaccine. In such a method embodiment, an immune response eliciting effective amount of a vaccine comprising a DNA construct operably encoding a VEGF receptor protein is administered to a patient having a growing tumor. Vaccination results in tumor growth arrest. Destruction of proliferating endothelial cells by the patient's immune system prevents vascularization of the tumor, in essence starving the tumor to death.

In the method embodiments of the present invention, the DNA vaccines can be administered enterally, such as by oral administration, or perenterally, such as by injection or intravenous infusion.

The vaccines of the present invention are useful for treatment and prevention of a number of disease states. For example, a patient suffering from a cancer, diabetic retinopathy, and the like, can benefit from immunization by the vaccines of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

In the Drawings, FIGURE 1 depicts the DNA sequence encoding human KDR, SEQ ID NO: 1;

FIGURE 2 depicts the protein sequence of human KDR, SEQ ID NO: 2;

FIGURE 3 depicts the DNA sequence encoding human Flt-1,SEQ ID NO: 3;

FIGURE 4 depicts the protein sequence of human Flt-1,SEQ ID NO: 4.;

FIGURE 5 depicts the DNA sequence encoding mouse Flk-1,SEQ ID NO: 5;

FIGURE 6 depicts the protein sequence of human Flk-1,SEQ ID NO: 6.;

FIGURE 7 is a pictorial representation of mouse lungs having varying levels of tumor coverage ranging from >50 % coverage (labeled 3) to <10 % coverage (labeled 1);

FIGURE 8 is a graphical representation of data demonstrating that mice vaccinated with a DNA vaccine of the invention (solid, heavy black line) and challenged by intravenous injection of CT-26 colon carcinoma cells, exhibited significantly reduced mortality relative to two control groups of mice (naive mice: solid thin line; control vaccine: dash-dot line);

FIGURE 9 is a graphical representation of data demonstrating the suppression of D121 Lewis lung carcinoma tumor growth in mice vaccinated with a DNA vaccine of the invention (pcDNA3.1-FLK-1) relative to two control groups of mice;

FIGURE 10 is a graphical representation of data demonstrating the suppression of B16 melanoma tumor growth in mice vaccinated with a DNA vaccine of the invention (●) relative to a control group (○); and

FIGURE 11 is a graphical representation of data demonstrating the upregulation of CD25, CD69, and CD2 positive CD8+ T cells in mice vaccinated with a DNA vaccine of the invention relative to a control group of mice.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

A DNA vaccine effective for inhibiting endothelial cell proliferation comprises a DNA construct that operably encodes a vascular

endothelial growth factor (VEGF) receptor protein. The term "DNA construct" as used herein and in the appended claims means a synthetic DNA structure that can be transcribed in target cells. The construct can comprise a linear nucleic acid such as a purified DNA, or preferably, DNA incorporated in a plasmid vector. The DNA can also be incorporated in a viral or bacterial vector, preferably an attenuated viral or bacterial vector that is non-pathogenic. Suitable DNAs are those that encode a VEGF receptor protein such as VEGFR-2 (KDR; SEQ ID NO: 2), VEGFR-1 (Flt-1; SEQ ID NO: 4), and Flk-1 (SEQ ID NO: 6), e.g., DNA sequences SEQ ID NO: 1, SEQ ID NO: 3, and SEQ ID NO 5, respectively.

Five VEGF sub-types have been identified, including VEGF-1 (also known as VEGF-A), VEGF-2 (also known as VEGF-C), VEGF-B, VEGF-D and VEGF-E. See, for example, U.S. Patent No. 6,235,713 to Achen *et al.* and references cited therein. VEGF receptors are protein-tyrosine kinases specific to endothelial cells. Several receptor protein-tyrosine kinases that are specific to endothelial cells have been identified, including Flt-1 (VEGF receptor 1; VEGFR-1), KDR (VEGFR-2), Flk-1 (the murine homolog of KDR), Flt-4 (VEGFR-3), Tie, Tie-2 and Tek, several of which are VEGF receptors.

The DNA vaccines of the present invention stimulate formation of CTLs that are active against proliferating endothelial cells, which overexpress VEGFR-2. Because VEGF receptors are only substantially expressed on proliferating endothelial cells, a CTL that forms in response to the vaccine will substantially target only tissues where active angiogenesis (e.g., vascularization) is occurring. Non-proliferating endothelial cells, such as the linings of established blood vessels, are substantially lacking in VEGF receptor antigens and are thus not affected by a CTL elicited by the vaccine.

In a preferred embodiment, the DNA vaccine comprises a polynucleotide sequence that operably encodes a VEGF receptor protein. This vaccine can promote activation of naive T cells, both directly and indirectly, through the intervention of dendritic cells.

As used herein, the term "immunity" refers to long term immunological protection against the virulent form of the infectious agent or

tumor antigen. The term "immunization" refers to prophylactic exposure to an antigen of a pathogenic agent derived from a non-virulent source, which results in immunity to the pathogen in the treated subject.

A DNA construct of the present invention preferably comprises a nucleotide sequence that encodes a VEGF receptor protein operably linked to regulatory elements needed for gene expression.

Useful DNA constructs preferably include regulatory elements necessary for expression of nucleotides. Such elements include, for example, a promoter, an initiation codon, a stop codon, and a polyadenylation signal. In addition, enhancers are often required for expression of a sequence that encodes an immunogenic target protein. As is known in the art, these elements are preferably operably linked to the sequence that encodes the desired protein. Regulatory elements are preferably selected that are operable in the species to which they are to be administered.

Initiation codons and stop codons are preferably included as part of a nucleotide sequence that encodes the VEGF receptor protein in a genetic vaccine of the present invention. The initiation and termination codons must be in frame with the coding sequence.

Promoters and polyadenylation signals included in a vaccine of the present invention are preferably selected to be functional within the cells of the subject to be immunized.

Examples of promoters useful in the vaccines of the present invention, especially in the production of a genetic vaccine for humans, include but are not limited to promoters from Simian Virus 40 (SV40), Mouse Mammary Tumor Virus (MMTV) promoter, Human Immunodeficiency Virus (HIV) such as the HIV Long Terminal Repeat (LTR) promoter, Moloney virus, Cytomegalovirus (CMV) such as the CMV immediate early promoter, Epstein Barr Virus (EBV), Rous Sarcoma Virus (RSV) as well as promoters from human genes such as human actin, human myosin, human hemoglobin, human muscle creatine, and human metalothionein.

Examples of polyadenylation signals useful in the vaccines of the present invention, especially in the production of a genetic vaccine for humans,

include but are not limited to SV40 polyadenylation signals and LTR polyadenylation signals.

In addition to the regulatory elements required for DNA expression, other elements can also be included in the DNA molecule. Such additional elements include enhancers. The enhancer can be, for example, human actin, human myosin, human hemoglobin, human muscle creatine and viral enhancers such as those from CMV, RSV and EBV.

Regulatory sequences and codons are generally species dependent, so in order to maximize protein production, the regulatory sequences and codons are preferably selected to be effective in the species to be immunized. One having ordinary skill in the art can produce DNA constructs that are functional in a given subject species.

The DNA constructs of the present vaccines can be "naked" DNA as defined in Restifo *et al. Gene Therapy* **7**, 89-92 (2000), the pertinent disclosure of which is incorporated by reference. Alternatively, the DNA can be operably incorporated in a vector. Useful delivery vectors include biodegradable microcapsules, immuno-stimulating complexes (ISCOMs) or liposomes, and genetically engineered attenuated live vectors such as viruses or bacteria.

Examples of suitable attenuated live bacterial vectors include *Salmonella typhimurium*, *Salmonella typhi*, *Shigella, Bacillus*, *Lactobacillus*, *Bacille Calmette-Guerin (BCG)*, *Escherichia coli*, *Vibrio cholerae*, *Campylobacter*, or any other suitable bacterial vector, as is known in the art. Methods of transforming live bacterial vectors with an exogenous DNA construct are well described in the art. See, for example, Joseph Sambrook and David W. Russell, *Molecular Cloning, A Laboratory Manual*, 3rd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2001).

Preferred viral vectors include Bacteriophages, Herpes virus, Adenovirus, Polio virus, Vaccinia virus, and Avipox. Methods of transforming viral vector with an exogenous DNA construct are also well described in the art. *See* Sambrook and Russell, above.

Liposome vectors are unilamellar or multilamellar vesicles, having a membrane portion formed of lipophilic material and an interior aqueous

portion. The aqueous portion is used in the present invention to contain the polynucleotide material to be delivered to the target cell. It is generally preferred that the liposome forming materials have a cationic group, such as a quaternary ammonium group, and one or more lipophilic groups, such as saturated or unsaturated alkyl groups having about 6 to about 30 carbon atoms. One group of suitable materials is described in European Patent Publication No. 0187702, and further discussed in U.S. Patent No. 6,228,844 to Wolff *et al.*, the pertinent disclosures of which are incorporated by reference. Many other suitable liposome-forming cationic lipid compounds are described in the literature. See, e.g., L. Stamatatos, *et al.*, *Biochemistry* 27:3917-3925 (1988); and H. Eibl, *et al.*, *Biophysical Chemistry* 10:261-271 (1979). Alternatively, a microsphere such as a polylactide-coglycolide biodegradable microsphere can be utilized. A nucleic acid construct is encapsulated or otherwise complexed with the liposome or microsphere for delivery of the nucleic acid to a tissue, as is known in the art.

The method aspects of the present invention comprise the step of administering DNA polynucleotides to tissue of a mammal, such as a human. In some preferred embodiments, the DNA polynucleotides are administered orally, intramuscularly, intranasally, intraperitoneally, subcutaneously, intradermally, or topically.

In a method aspect of the present invention, a DNA vaccine can be utilized to provide long term inhibition of endothelial cell proliferation in a patient treated with the vaccine. In one preferred method embodiment, a DNA vaccine comprising a polynucleotide construct operably encoding a VEGF receptor protein is administered to a mammal in need of inhibition of endothelial cell proliferation, in an amount that is sufficient to elicit an immune response against proliferating endothelial cells.

The present invention also provides a method of inhibiting angiogenesis in a mammal treated with the DNA vaccine. In such a method embodiment, a vaccine comprising a DNA construct operably encoding a VEGF receptor protein is administered to a mammal suffering from an angiogenesis related disease, in an amount sufficient to elicit an immune response against proliferating endothelial cells.

In yet another method aspect of the present invention, tumor growth is inhibited by treatment of a mammal with a DNA vaccine. In such a method embodiment, an immune response eliciting amount of a vaccine comprising a DNA construct operably encoding a VEGF receptor protein is administered to a mammal having a growing tumor. Treatment with the vaccine results in tumor growth arrest by immunizing the mammal against proliferating endothelial cells. Destruction of proliferating endothelial cells by the mammal's immune system prevents, or at least minimizes vascularization of the tumor.

In the method embodiments of the present invention, the vaccines can be administered enterally, such as by oral administration, or by intramuscular injection. Preferably, the mammal treated with the inventive vaccine is a human. A patient suffering from cancer, such as lung or colon carcinoma, or prostate tumors, diabetic retinopathy, and the like, can benefit from immunization by the vaccines of the present invention.

Vaccines of the present invention are preferably formulated with pharmaceutically acceptable carriers or exipients such as water, saline, dextrose, glycerol, and the like, and combinations thereof. The vaccines can also contain auxiliary substances such as wetting agents, emulsifying agents, buffers, and the like.

The vaccines of the present invention are preferably administered orally to a mammal, such as a human, as a solution or suspension in a pharmaceutically acceptable carrier, at a DNA concentration in the range of about 1 to about 10 micrograms per milliliter. The appropriate dosage will depend upon the subject to be vaccinated, and in part upon the judgment of the medical practitioner administering or requesting administration of the vaccine.

The vaccines of the present invention can be packaged in suitably sterilized containers such as ampules, bottles, or vials, either in multi-dose or in unit dosage forms. The containers are preferably hermetically sealed after being filled with a vaccine preparation. Preferably, the vaccines are packaged in a container having a label affixed thereto, which label identifies the vaccine, and bears a notice in a form prescribed by a government agency such as the United States Food and Drug Administration reflecting approval of the vaccine under

appropriate laws, dosage information, and the like. The label preferably contains information about the vaccine that is useful to an health care professional administering the vaccine to a patient. The package also preferably contains printed informational materials relating to the administration of the vaccine, instructions, indications, and any necessary required warnings.

Preferably, the vaccines for the present invention comprise DNA constructs that encode one or more VEGF receptor proteins, such as tyrosine kinases that are specific to endothelial cells, including, for example Flt-1, KDR, Flk-1, and functional homologs thereof. The functional homologs preferably share at least about 80 % homology with the aforementioned VEGF receptor proteins.

The amino acid sequences of VEGF receptor proteins have been disclosed in the art, as have the nucleic acid sequences encoding these proteins. The nucleic acid sequence encoding KDR (FIG. 1, SEQ ID NO: 1), and its corresponding protein sequence (FIG. 2, SEQ ID NO: 2) have been published by Yu *et al.*, in the *EMBL* database of the European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SD, UK (EMBL accession number is EMBL:AF063658), the disclosure of which is incorporated herein by reference. The nucleic acid sequence encoding Flt-1 (FIG. 3, SEQ ID NO: 3) , and its corresponding protein sequence (FIG. 4, SEQ ID NO: 4) have been published by Yu *et al.*, in the *EMBL* database of the European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SD, UK (EMBL accession number is EMBL:AF063657), the disclosure of which is incorporated herein by reference. The nucleic acid sequence encoding Flk-1, and its corresponding protein sequence have been published by Mathews *et al.*, *Proc. Natl. Acad. Sci. USA* 1991, 88:9026-9030, and the structures were corrected by Quinn *et al.*, *Proc. Natl. Acad. Sci. USA* 1991, 90:7533-7537, the relevant disclosures of which are incorporated herein by reference. The corrected DNA sequence of Flk-1 is provided in FIG 5 as SEQ ID NO: 5, and the corrected protein sequence of Flk-1 is provided in FIG. 6 as SEQ ID NO:6.

Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence to VEGF receptor proteins such as KDR, Flk-1 and Flt-1, can be used in the practice of the invention. Such DNA sequences include those which are capable of hybridizing to the VEGF receptor sequences as well. Preferably the functionally equivalent homologs of the VEGF receptor protein DNA shares at least about 80 % homology with the DNA encoding the aforementioned VEGF receptor proteins.

Altered DNA sequences which can be used in accordance with the invention include deletions, additions or substitutions of different nucleotide residues resulting in a sequence that encodes the same or a functionally equivalent gene product. The gene product itself may contain deletions, additions or substitutions of amino acid residues within the VEGF receptor sequences, which result in a silent change, thus producing a functionally equivalent VEGF receptor proteins. Such amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; amino acids with uncharged polar head groups having similar hydrophilicity values include the following: leucine, isoleucine, valine; glycine, alanine; asparagine, glutamine; serine, threonine; phenylalanine, tyrosine. As used herein, a functionally equivalent VEGF receptor refers to a receptor that binds to VEGF or fragments thereof, but not necessarily with the same binding affinity of its counterpart native KDR, Flk-1 or Flt-1.

The DNA sequences of the invention may be engineered in order to alter the VEGF receptor coding sequence for a variety of ends including, but not limited to, alterations that modify processing and expression of the gene product. For example, mutations may be introduced using techniques that are well known in the art, e.g. site-directed mutagenesis, to insert new restriction sites, to alter glycosylation patterns, phosphorylation, etc.

Mouse Flk-1 (SEQ ID NO: 6) shares an approximately 85% homology with human KDR (SEQ ID NO: 2) and plays an analogous role in

mouse physiology to the role of KDR in humans. In fact, VEGFR-2 is often referred to as KDR/Flk-1, reflecting the close analogy between these two VEGF receptor homologs. For this reason, treatment of mice with a DNA vaccine of the invention, encoding Flk-1 (e.g., DNA SEQ ID NO: 5) was chosen as a suitable model for human DNA vaccines encoding KDR.

The following examples are provided to further illustrate the features and embodiments of the present invention, and are not meant to be limiting.

Materials, Methods and Examples.

Materials. C57/BL/6J and Balb/C mice were obtained from the Scripps Research Institute breeding facility. The murine tumor cell lines used for evaluation included the melanoma cell line B16 and the colon carcinoma cell line CT26, all of which were obtained from Dr. I. J. Fidler, MD Anderson Cancer Center, Houston, TX. The murine Lewis lung cancer cell line D121 was obtained from Dr. Lea Eisenbach, Weizmann Institute, Rehovot, Israel. The DNA encoding Flk-1 was kindly provided by Dr. Lemischka (Princeton University, Princeton, NJ), and was cloned into the pcDNA3.1 eucaryotic expression vector provided by Invitrogen, Huntsville, Alabama, using the restriction sites KpnI and XbaI. An attenuated strain of *Salmonella typhimurium* was provided by B.A.D. Stocker (Stanford University, Stanford, CA). Antibodies were obtained from BD Biosciences, Bedford, MA. T-STIM culture supplement was obtained from BD Biosciences, Bedford, MA. Fluorescein isothiocyanate (FITC) and R-Phycoerythrin (PE) were obtained from Molecular Probes, Eugene, OR. FITC-labeled and PE-labeled antibodies were prepared according to the manufacturer's recommended protocols.

EXAMPLE 1. Preparation of a DNA Vaccine Encoding Flk-1.

The pcDNA3.1 vector containing Flk-1 DNA (SEQ ID NO: 5; about 10 pg to about 0.1 μg of pDNA) was electroporated into freshly prepared attenuated *Salmonella typhimurium*, utilizing a Bio-Rad Pulser at 2.5 kV, 25 μF, and 200 Ohm according to the manufacturer's recommended procedures. *Salmonella* containing the vector were selected on ampicillin-containing plates. Colonies were picked the next day and cultured overnight in LB broth (EM

Science, Gibbstown, NJ) with ampicillin added. The bacteria were isolated and washed in phosphate buffered saline (PBS). The washed bacteria were then suspended in PBS medium at a concentration of about 1 x 10^9 recombinant *Salmonella* per milliliter of PBS, to form a vaccine solution for later use. The vaccine was stored in sealed ampules until used. A "control vaccine" consisting of *Salmonella* transformed with the pcDNA3.1 vector alone (no Flk-1 DNA) was also prepared according to the same procedure. The plasmid DNA was stored at about -80 °C before transforming the *Salmonella*.

EXAMPLE 2. Vaccination of Mice with a DNA Vaccine Encoding Flk-1.

Balb/C mice (about 6 mice per treatment group) were vaccinated with the DNA vaccine of Example 1 (about 1 x 10^8 recombinant *Salmonella* in about 100 μl of PBS) by oral gavage, three times at two week intervals. Another group of mice were vaccinated with control vaccine (consisting of attenuated *Salmonella* containing the empty vector) according to the same schedule as the mice vaccinated with the inventive vaccine.

EXAMPLE 3. Evaluation of Tumor Resistance of Vaccinated Mice.

About two weeks after the third vaccination, Balb/C mice from Example 2 (about 6 mice per treatment group) were challenged with either about 1 x 10^5 B16 melanoma cells (subcutaneously), about 1 x 10^5 D121 Lewis lung carcinoma cells (subcutaneously), or about 7.5 x 10^4 CT26 colon carcinoma cells (intravenously). The subcutaneous Lewis lung tumors were surgically removed after about two weeks of growth to allow spontaneous dissemination to the lung. Subcutaneous tumor growth was measured in two dimensions every other day, and tumor volume was calculated according to the formula:

$$\text{volume} = (\text{width}^2)(\text{length} \div 2)$$

for each tumor. The amount of spontaneous metastasis of D121 to the lungs was evaluated about 30 days after removal of the subcutaneous primary tumor. The mice were sacrificed and necropsied, and the tumor burdens of the lungs were evaluated according to the percentage of the lung surface that was covered by tumor and scored as "0" for no tumor, "1" for less than about 20% tumor coverage, "2" for about 20 to about 30% tumor coverage, and "3" for greater than about 50% tumor coverage. FIG. 7 shows pictures of lungs from three mice

challenged with D121 Lewis lung carcinoma cells. The lower lung was scored 1, whereas the upper two lungs were scored 3, having a large proportion of the lung surface covered by tumors. Animals that died prior to the 30 day evaluation were given a "+" score.

The results of these evaluations are provided in Tables 1-4, and in FIGS. 8-10, discussed in detail below.

Table 1. Tumor Metastasis in Balb/C Mice Challenged with D121 Lewis Lung Carcinoma Cells.

Mouse Vaccination Group	Metastatic Scores
Control - vaccination with untransformed Salmonella	3,3,3,3,+,+
Control - vaccination with control vaccine (containing empty vector)	3,3,3,3,+,+
Vaccination with DNA Vaccine of Example 1 (containing Flk-1)	0,0,1,1,1,2,2

The Balb/C mice that were challenged by intravenous injection of CT-26 colon carcinoma cells were evaluated for mortality over about a 63 day (7 week) period. Mortality information is presented in Table 2 below, and graphically illustrated in FIG. 8.

In FIG. 8, the % survival of mice treated with the inventive vaccine of Example 1 is indicated by the heavy, solid line at 100% survival. The % survival of naive mice (no vaccination) challenged with the C26 cell is indicated by the solid, thin line, whereas, the % survival of the mice treated with the control vaccine (no Flk-1 DNA) is indicated by the dot-dash line.

Table 2. Suppression of Mortality in Balb/C Mice Immunized With the Vaccine of Example 1 and Challenged with CT 26 Carcinoma.

Treatment	% Survival on Day 30	% Survival on Day 36	% Survival on Day 63
Control, No Vaccine	50	0	0
Control Vaccine	33	0	0
Vaccine of Ex. 1	100	100	100

The suppression of growth of the primary (subcutaneous) tumor in D121 challenged Balb/C mice was evaluated by determination of primary tumor volume at day 14 after challenge. Results are presented in Table 3 below, and graphically illustrated in FIG. 9.

In FIG. 9, the first bar, labeled "PBS" indicates mice that were not vaccinated (naive mice), the middle bar, labeled "empty vector" indicates mice treated with the control vaccine, and the third bar, labeled "pcDNA3.1-FLK1" indicates mice immunized with the inventive vaccine of Example 1.

Table 3. Suppression of Subcutaneous D121 Carcinoma Tumor in Balb/C Mice Immunized With the Vaccine of Example 1.

Treatment	Tumor volume mm^3	Standard Deviation
Control No Vaccine	665	227
Control Vaccine	641	157
Vaccine of Ex. 1	183	35

Suppression of subcutaneous B16 melanoma tumor growth was evaluated by monitoring the subcutaneous tumor volume over a period of about 17 days after tumor challenge. Results are presented in Table 4 and graphically illustrated in FIG. 10 below. In FIG. 10, average tumor volume data indicated

by (●) represents mice immunized with the inventive vaccine of Example 1, whereas data indicated by (○) indicates mice treated with the control vaccine.

Table 4. Suppression of Subcutaneous B16 Melanoma Tumor in Balb/C Mice Immunized With the Vaccine of Example 1.

	Tumor Volume (mm^3) on Day			
Treatment	0	9	14	17
Control Vaccine	0	907	1273	4213
Vaccine of Ex. 1	0	447	462	1063
% Tumor Suppression	---	51%	64%	75%

EXAMPLE 4. Upregulation of CD25, CD69 and CD2 Activation Markers in Splenocytes (CD8+ T Cells) From Vaccinated Mice.

C5/7BL/6J mice (about 4 mice per treatment group) were vaccinated with the DNA vaccine of Example 1 and the control vaccine (no Flk-1) as described in Example 2. Splenocytes were isolated from the immunized mice and the control mouse group about six weeks after the third vaccination. The splenocyte cells were cultured for 24 hours together with cells from a B16 melanoma cell line transduced to express Flk-1 and with untransformed B16 cells in T cell medium (about 5 mL per culture) containing about 4% by volume of T-STIM culture supplement (Cat. # 354115, BD Biosciences, Bedford, MA). The cells were then stained with FITC-conjugated CD8+ antibody and PE-conjugated antibodies of CD25, CD69, and CD2. The cell suspensions were evaluated using a Becton Dickenson FAC scan to determine the percentage of CD8+T cells positive for CD25 and CD69 for each splenocyte/B16 melanoma cell combination. The results are presented in Table 5 and are illustrated graphically in FIG. 11.

Table 5. Upregulation of CD25, CD69 and CD2 Activation Markers in Splenocytes From Vaccinated Mice

Treatment	% CD25 positive	% CD69 positive	CD2 positive mean fluorescence
Control vaccine + B16-Flk-1 cells	9	18	570 mfu
DNA vaccine + B16 cells	12	29	550 mfu
DNA vaccine + B16-Flk-1 cells	21	35	700 mfu

mfu = mean fluorescence units.

The results provided in Tables 1-5 and FIGS. 8-11 demonstrate that the DNA vaccine of Example 1, comprising a DNA encoding Flk-1, the murine analog of KDR, can effectively immunize mice against a variety of tumor forming cancer cells. Although not intending to be bound by theory, it is believed that the vaccine acts by inhibiting angiogenesis in the tumor, i.e, preventing new blood vessel formation and effectively starving the tumor.

The data in Table 1 demonstrate that the inventive vaccine of Example 1 leads to a suppression of tumor metastasis to the lungs of mice challenged with D121 Lewis lung carcinoma. None of the mice immunized with the vaccine of Example 1 died, and all had less than about 50% tumor coverage on the lungs (2 had <20%). In contrast, two mice died from each control group and all of the remaining mice had greater than about 50% tumor coverage on the lungs.

The inventive vaccine of Example 1 also significantly decreased mortality of Balb-C mice that were challenged intravenously by CT-26 colon carcinoma cells, as demonstrated by the data in Table 2 and FIG. 8. All of the mice immunized with the vaccine of Example 1 survived the entire 63 day

observation period after challenge. In the control groups, however, all of the mice had died by day 36 post challenge.

As demonstrated by the data in Table 3 and FIG. 9, subcutaneous D121 Lewis lung carcinoma tumor growth was suppressed by immunization with the inventive vaccine of Example 1 by a factor of about 4.3 to about 4.5, relative to the control mouse groups treated with no vaccine or the control vaccine..

Similarly, as shown in Table 4 and FIG. 10, subcutaneous B16 melanoma tumor growth was suppressed by a factor of about 4 in mice immunized with the inventive vaccine of Example 1, relative to tumor growth in the control group.

The data in Table 5 and FIG. 11 show that splenocytes isolated from C57/BL/6J mice vaccinated with the DNA vaccine of Example 1 exhibited an upregulation of CD2, CD25 and CD69 activation markers relative to the control group of mice, when cultured with B16 melanoma cells transformed to present Flk-1 antigen.

Numerous variations and modifications of the embodiments described above can be effected without departing from the spirit and scope of the novel features of the invention. It is to be understood that no limitations with respect to the specific embodiments illustrated herein are intended or should be inferred. It is, of course, intended to cover by the appended claims all such modifications as fall within the scope of the claims.